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Low Energy Laser Irradiation Fails to Modulate the Inflammatory Function of Human Monocytes and Endothelial Cells

Maarten G. Bouma, MD, Wim A. Buurman, PhD, and Frans A.J.M. van den Wildenberg, PhD

Department of Surgery, University of Limburg, 6200 MD Maastricht, The Netherlands

Background and Objective: **In view of the important regulatory role of cytokines in wound healing and inflammation, we investigated the effects of low energy laser irradiation on cytokine release by human peripheral blood monocytes (MΦ) and human umbilical vein endothelial cells (HUVEC) in vitro. Also, the effects of laser light on the expression of endothelial adhesion molecules, another important feature of inflammatory and regenerative responses, were assessed.**

Study Design/Materials and Methods: **Cells were irradiated with a pulsed GaAs-laser (904 nm) at energy densities 0 (=sham), 0.3, 3.0, or 9.0 J/cm^2 and subsequently incubated in absence or presence of endotoxin (MΦ) or the proinflammatory cytokines TNFα and IL-1β (HUVEC).**

Results: **Irradiation at any of the dosages used did not significantly affect spontaneous or endotoxin-induced release of TNFα, IL-6, and IL-8 by MΦ. Similarly, secretion of IL-6 and IL-8 by resting or cytokine-activated HUVEC after either single or repeated laser treatment was unchanged as compared to sham-irradiated controls. Moreover, laser treatment did not induce de novo expression or upregulation of the endothelial adhesion molecules E-selectin, ICAM-1, and VCAM-1, and it failed to modify their expression in response to stimulation with TNFα or IL-1β.**

Conclusion: **We conclude that with the specific laser parameters and dose-regimen used, low energy laserlight does not affect the inflammatory function of human monocytes and endothelial cells in vitro.** © 1996 Wiley-Liss, Inc.

Key words: adhesion molecules, biostimulation, cytokines, endothelium, Ga-As laser, inflammation, mononuclear phagocytes

INTRODUCTION

Low energy laser therapy has been applied clinically for stimulation of wound healing and the treatment of a variety of inflammatory soft tissue conditions [1,2]. The effects of low energy laser irradiation on the regulation of inflammatory responses at the cellular level are, however, still not well characterized [3–5].

Inflammatory responses are the result of a complex interplay between a variety of immune and nonimmune cells. Over the last decades cytokines have been identified as crucial mediators of tissue repair and inflammation [6]. Cytokines are produced by a wide array of cell types, such as mononuclear phagocytes, lymphocytes, and endothelial cells, and serve as communication signals among immunocompetent cells as well as between immune cells and connective tissue cells. Specifically, cytokines are involved in chemotaxis, adhesion to vascular endothelium, and subsequent transendothelial migration of leukocytes

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Address reprint requests to Maarten G. Bouma, M.D., Department of Surgery, University of Limburg (Fac. II), P.O. Box 616, 6200 MD Maastricht, The Netherlands.

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into target tissue, events that are crucial in the development of an inflammatory response [7,8]. When activated by the mononuclear cell-derived proinflammatory cytokines Interleukin-1 (IL-1) and Tumor Necrosis Factor-α (TNFα), endothelial cells produce chemotactic cytokines such as IL-8 and express various adhesion molecules for leukocytes, thereby facilitating leukocyte extravasation [9–11].

In addition to their role in inflammation, cytokines are of importance in tissue repair. Mononuclear cell-derived TNFα promotes fibroblast proliferation and collagen synthesis [12–16], and furthermore, TNFα and IL-8 are known to mediate angiogenesis [17,18].

The objectives of this study were to determine whether low energy laser irradiation induces release of cytokines by monocytes and endothelial cells and alters cytokine release by monocytes and endothelial cells in response to activation with inflammatory mediators, such as lipopolysaccharide (LPS) and the proinflammatory cytokines IL-1β and TNFα. In addition, we investigated whether laser irradiation affected the expression of different types of adhesion molecules by either resting or cytokine-stimulated endothelial cells.

MATERIALS AND METHODS

Cell Isolation and Culture

Buffy coats, obtained from healthy blood donors after informed consent for research was given, were kindly provided by the Red Cross Blood Bank Zuid Limburg (Maastricht, The Netherlands). Human peripheral blood monocytes (MΦ) were isolated from buffy coats, essentially according to the method described by Graziano and Fanger [19]. Briefly, mononuclear cell suspensions obtained after density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) were allowed to clump by low speed centrifugation at 4°C. Cell clumps, consisting for 80–95% of monocytes, were separated by sedimentation through ice-cold bovine calf serum (BCS, Hyclone Laboratories, Logan, UT) resulting in cell suspensions consisting of >85% of monocytes.

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords by collagenase treatment and were seeded into fibronectin-coated (fibronectin kindly provided by Dr. J. van Mourik, CLB, Amsterdam, The Netherlands) tissue-culture flasks (Costar Corp., Cambridge, MA) in complete medium consisting of RPMI 1640 supplemented with 50 μg/ml heparine (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 100 IU penicillin, 100 μg/ml streptomycin, 30 μg/ml Endothelial Cell Growth Supplement (Collaborative Research, Bedford, MA), 10% BCS, and 10% human serum (HS). Confluent HUVEC monolayers were serially passed every 3–4 days by trypsinization. For experiments, HUVEC passage 2–3 were plated onto fibronectin-coated, 96-well flat-bottom tissue culture plates (Costar) in complete media 24 hours prior to laser irradiation.

Laser Irradiation

MΦ were resuspended in RPMI and plated on 96-well, flat-bottom tissue culture plates at 1×10^5 cells per well to a final volume of 50 μL. Confluent HUVEC monolayers in 96-well, flat-bottom tissue culture plates were rinsed once with RMPI, and subsequently 50 μL medium was added to each well.

Cell cultures were irradiated using an Phy-Action 796 device with a 15W model 241 probe (Uniphy, Eindhoven, The Netherlands). This is an 904 nm GaAs diode-laser with the following physical parameters: average power output: 11.25 mW; spot size at 1 cm distance: 0.28 cm^2; power density: 40.18 mW/cm^2; pulse frequency: 5,000 Hz; pulse width: 150 nsec; total angle of divergence: 21°. With the use of a specially designed positioning collar, the laser probe was positioned exactly centrally at an irradiation distance of 1 cm from the bottom of the well to be irradiated, thereby adjusting the spot size to the surface area of the well. Average power output was verified before every irradiation procedure using a built-in foto-electric test-eye.

Experimental Protocol

Under sterile conditions, cells were exposed to either a single sham irradiation or a single laser irradiation for 7.5, 75, or 225 sec, yielding energy densities of 0 J/cm^2 (= sham) and 0.3, 3.0, or 9.0 J/cm^2, respectively. In another series of experiments, HUVEC cultures were exposed to an irradiation dose, as described above, daily during three consecutive days (Fig. 1). Upon completion of the laser treatment, the medium overlying the cells was removed and 100 μL of fresh medium with or without activator was added to the wells. For MΦ, lipopolysaccharide (LPS, Sigma) was used as activator during a 20-hour incubation period following laser treatment. HUVEC were activated with the proinflammatory cytokines

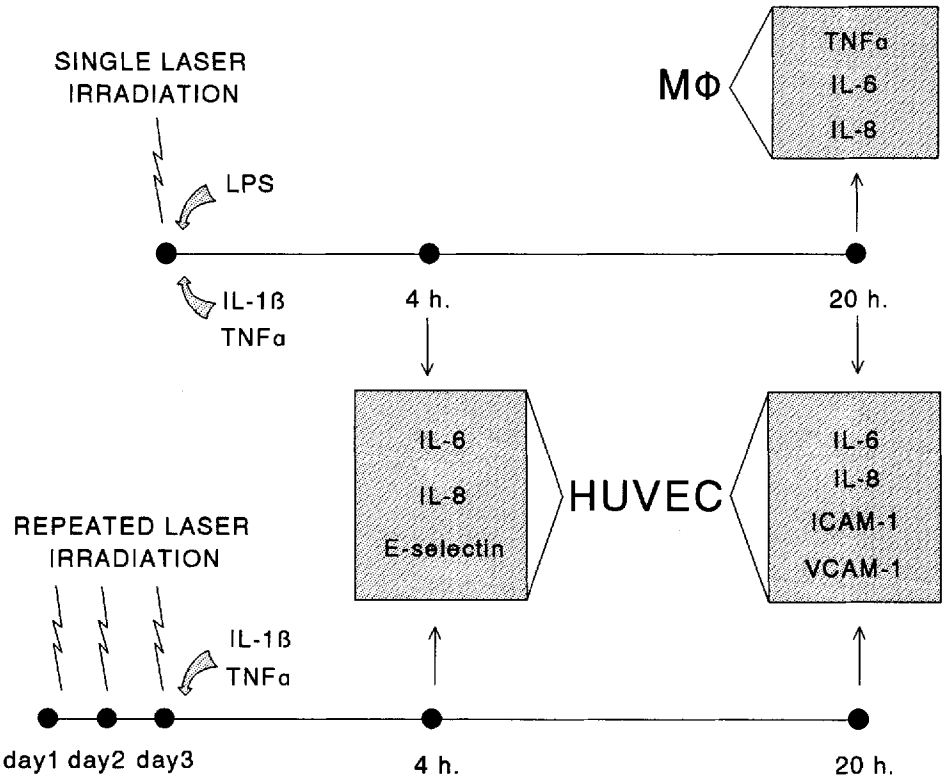

Fig. 1. Schematic representation of the experimental protocols for irradiation and subsequent stimulation of monocytes (MΦ) and HUVEC.

TNFα (BASF/Knoll, Ludwigshafen, Germany) or IL-1β (Immunex, Seattle, WA) as indicated during either 4 hours or 20 hours after irradiation. These two timepoints were selected to achieve maximal expression of the endothelial adhesion molecules E-selectin or ICAM-1 and VCAM-1, respectively [9]. At the end of the respective incubation periods, the culture supernatants were harvested and stored at −20° C until determination of cytokine concentration by ELISA. Endothelial monolayers were fixed for determination of adhesion molecule expression as described below. Cell viability, as determined by trypan blue exclusion, was not significantly affected by laser treatment with or without subsequent stimulation (data not shown).

Cytokine Assays

Cytokine concentrations in the culture supernatants were determined using previously described enzyme-linked immunosorbent assays (ELISAs) for TNFα [20], IL-6 [21], and IL-8 [22]. Each experimental sample was assayed at least in duplicate. The lower detection limits of the ELISAs were 1 pg/ml for TNFα, 10 pg/ml for IL-6, and 5 pg/ml for IL-8.

ELISAs for Endothelial Adhesion Molecules

Upon completion of the experimental protocol, endothelial monolayers were rinsed three times with phosphate-buffered saline (PBS) supplemented with calcium and magnesium and fixed with 0.025% glutaraldehyde for 10 minutes at room temperature. Finally, the fixed monolayers were rinsed five times with PBS, the wells were incubated with 1% bovine serum albumin in PBS, and plates were kept at 4°C until use in ELISA. Cell-surface expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) was detected by incubation for 2 hours at room temperature with mAbs ENA-1 (anti-E-selectin), RR-1 (anti-ICAM-1, a kind gift of Dr. R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT) and 1G11 (anti-VCAM-1, a kind gift of Dr. D.O. Haskard, Hammersmith Hospital, Department of Rheumatology, London, UK), respectively, followed by incubation with peroxi-

dase-conjugated, goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA) for 1 hour. TMB was added as substrate, and the reaction was stopped after 15 minutes with 1M H_2SO_4. Photospectrometry was performed at 450 nm.

Statistical Analysis

Data are presented as mean ± S.E.M. Results were analyzed statistically by Student' t-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effects of Laser Irradiation on Release of Cytokines by MΦ and Endothelial Cells

Unstimulated monocytes did not release detectable amounts of TNFα into the culture supernatants during a 20-hour incubation period following sham irradiation (Fig. 2A). At the same time a low basal production of IL-6 (2.30 ± 1.64 ng/ml) and IL-8 (1.32 ± 0.25 ng/ml) was observed (Fig. 2B,C). A single laser treatment at energy densities 0.3, 3.0 and 9.0 J/cm^2 did not induce detectable levels of TNFα, nor did irradiation alter the spontaneous release of IL-6 and IL-8. Activation of monocyte cultures with a low (5 ng/ml) or a high (500 ng/ml) dose of LPS following sham irradiation, induced a dose-dependent release of all three cytokines. Irradiation at 0.3 J/cm^2 did not alter the release of IL-6 and IL-8 by either submaximally or maximally stimulated monocytes. At this energy density, however, TNFα levels of the same supernatants were slightly enhanced as compared with sham-irradiated controls, although statistical significance was not reached (6.70 ± 0.87 vs. 5.11 ± 0.46 ng/ml for cells irradiated at 0.3 J/cm^2 vs. sham-irradiated cells, respectively). Irradiation at an energy density of 3.0 J/cm^2 also nonsignificantly enhanced TNFα release, whereas IL-6 release remained unaffected. In contrast, after treatment at dosages of 3.0 and 9.0 J/cm^2 a lower IL-8 production by monocytes was observed in response to submaximal stimulation with 5 ng/ml LPS (101.06 ± 29.18 and 96.25 ± 11.95 vs. 162.94 ± 67.55 ng/ml for cells irradiated at 3.0 and 9.0 J/cm^2 vs. sham-irradiated cells, respectively). These differences from sham-irradiated controls were found to be statistically nonsignificant. Similar effects on IL-8 release were obtained with cells activated with a 100-fold higher concentration of LPS.

Next, the effects of laser irradiation on cytokine release by endothelial cells were assessed.

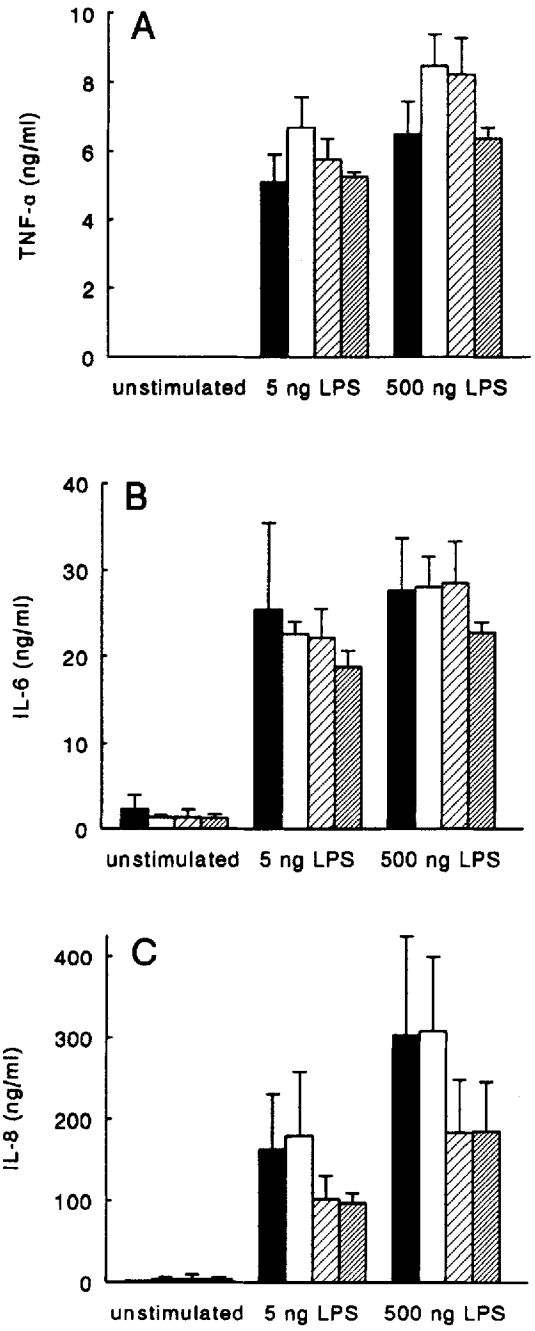

Fig. 2. Release of TNFα (A), IL-6 (B), and IL-8 (C) by unstimulated and LPS-stimulated monocytes following laser irradiation at energy densities 0 (solid bars), 0.3 (open bars), 3.0 (hatched bars), and 9.0 J/cm^2 (double-hatched bars). After irradiation, monocytes were incubated with or without 5 or 500 ng/ml LPS for 20 hours and subsequently cytokine release into the supernatants was determined by ELISA. Data are presented as mean ± SEM of three separate experiments.

During incubation for 4 hours (Fig. 3) or 20 hours (data not shown), unstimulated, sham-irradiated HUVEC spontaneously released small quantities

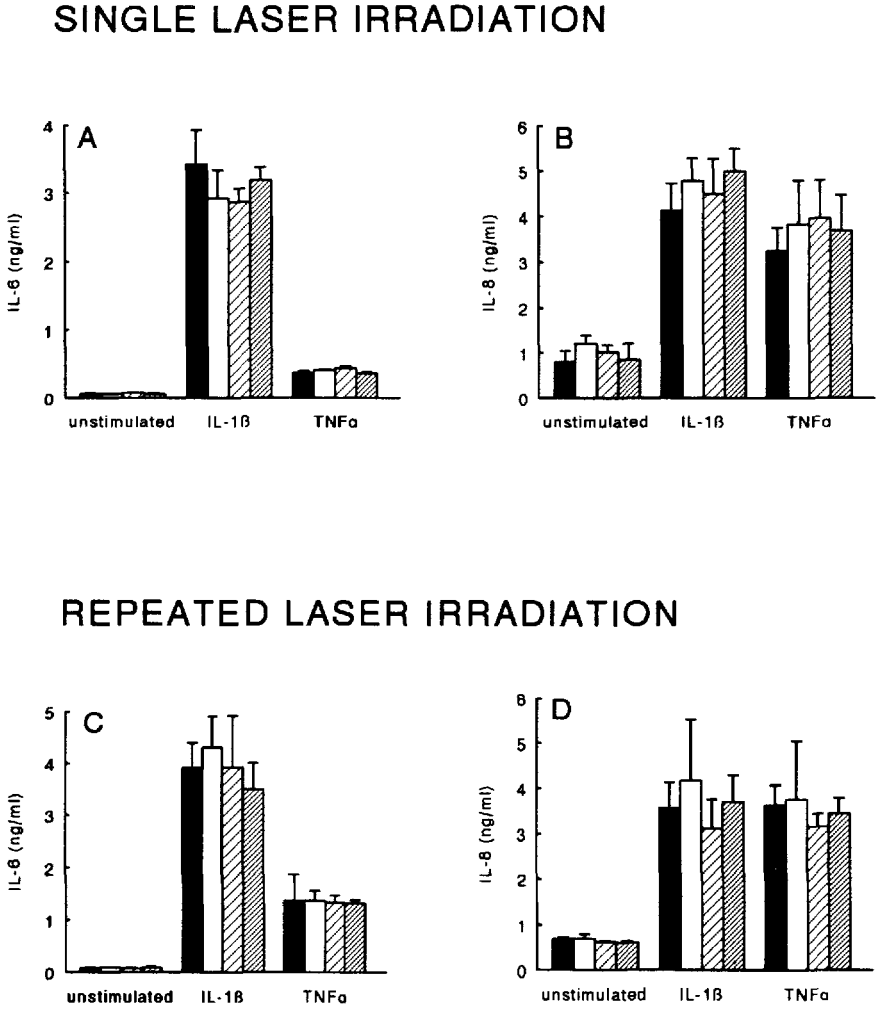

Fig. 3. Release of IL-6 (**A** and **C**) and IL-8 (**B** and **D**) by unstimulated and cytokine-activated HUVEC following single or repeated laser irradiation at energy densities 0 (solid bars), 0.3 (open bars), 3.0 (hatched bars), and 9.0 J/cm^2 (double-hatched bars). After irradiation, HUVEC were incubated with or without 10 U/ml IL-1β or 10 ng/ml TNFα for 4 hours, and subsequently cytokine release into the supernatants was determined by ELISA. Data are presented as mean ± SEM of four separate experiments.

of both IL-6 and IL-8. Secretion of both cytokines was upregulated during stimulation with IL-1β (10 U/ml) and, to a lesser extent, with TNFα (10 ng/ml). A single irradiation at any of the three energy densities used did not affect spontaneous as well as cytokine-induced release of both IL-6 and IL-8 during a 4-hour (Fig. 3A,B) and a 20-hour incubation period (data not shown). Next, the effects of repeated laser treatment on cytokine release by resting and activated HUVEC were investigated. During a 4-hour incubation after repeated laser irradiation at 0.3 to 9.0 J/cm^2 both spontaneous and cytokine-induced release of IL-6 (Fig. 3C) and IL-8 (Fig. 3D) remained unaltered, when compared with sham-irradiated controls. Release of both cytokines was also unaffected under these conditions during 20 hours of incubation (data not shown).

Effects of Laser Irradiation on Expression of Endothelial Adhesion Molecules

In parallel with the release of cytokines, the expression of specific adhesion molecules by sham-irradiated and light-treated HUVEC was determined. During a 4-hour incubation period following either single or repeated laser treatment, expression of E-selectin was identical in both irradiated and sham-irradiated groups (Fig. 4A,D). Moreover, laser treatment did not affect expression of E-selectin in response to stimula-

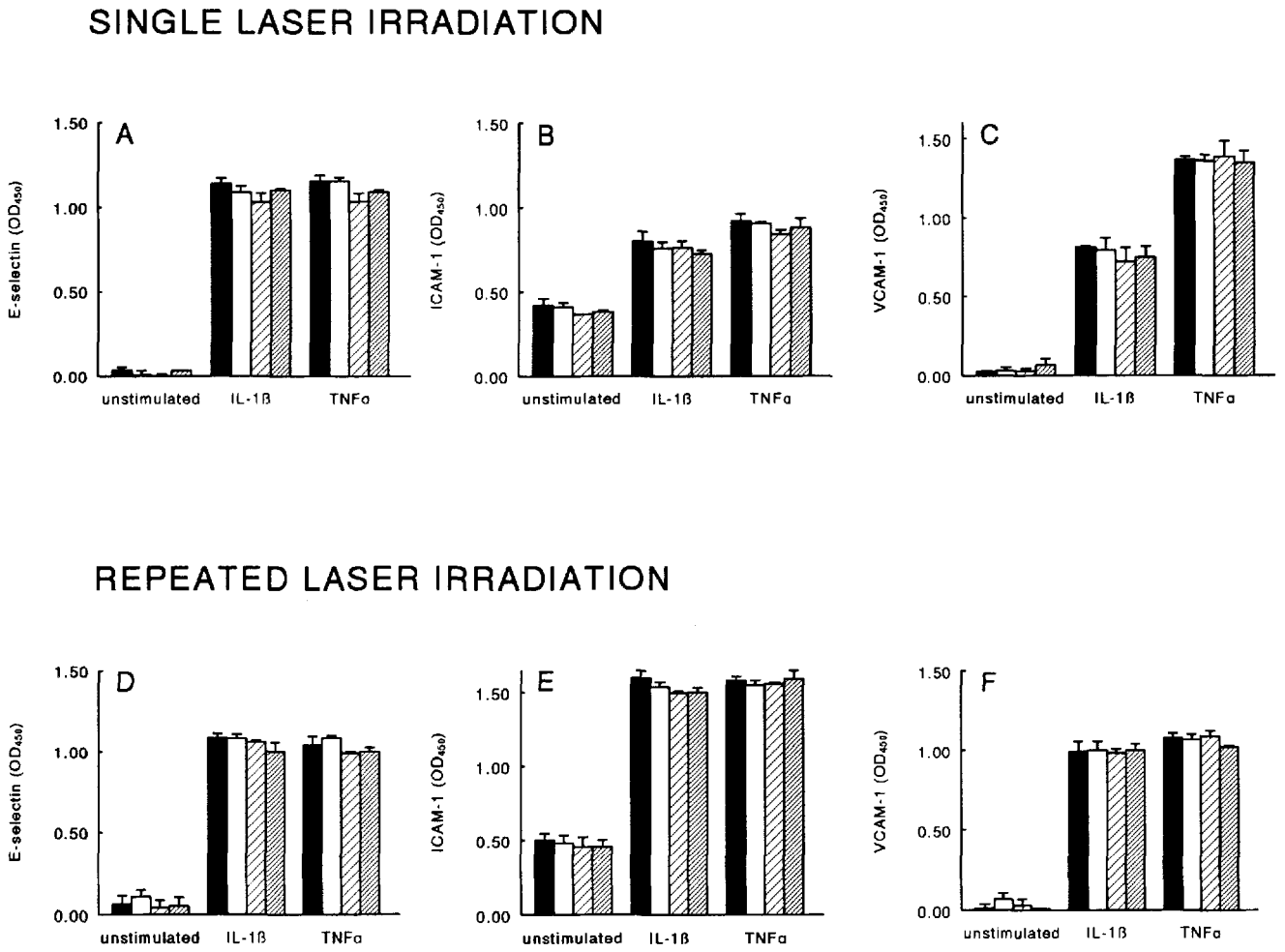

Fig. 4. Expression of the endothelial adhesion molecules E-selectin (**A** and **D**), ICAM-1 (**B** and **E**), and VCAM-1 (**C** and **F**) by unstimulated and cytokine-activated HUVEC following single or repeated laser irradiation at energy densities 0 (solid bars), 0.3 (open bars), 3.0 (hatched bars), and 9.0 J/cm^2 (double-hatched bars). After irradiation, HUVEC were incubated with or without 10 U/ml IL-1β or 10 ng/ml TNFα for 4 hours to induce E-selectin and for 20 hours to induce ICAM-1 and VCAM-1. Subsequently, cell-surface expression of the adhesion molecules was determined by ELISA. The results are expressed as optical densities measured by photospectrometry at 450 nm (OD_{450}). Data are presented as mean ± SEM of four separate experiments.

tion with IL-1β or TNFα. ICAM-1, which is constitutively expressed by endothelial cells [24], was neither upregulated nor inhibited by laser irradiation of resting HUVEC (Fig. 4B,E). Cytokine-induced upregulation of this expression was identical in all experimental groups. Finally, although after single or repeated light irradiation no induction of VCAM-1 on unstimulated HUVEC could be observed, laser treatment also failed to modulate VCAM-1 expression in response to stimulation with cytokines during 20 hours of incubation (Fig. 4C,F).

DISCUSSION

During the last three decades, the clinical application of low energy laser therapy to enhance wound healing and to reduce inflammatory activity in a variety of acute and chronic conditions has steadily increased, particularly in Europe. Nevertheless, considerable skepticism toward the concept of laser-induced biostimulation exists within the medical profession, and approval from the Food and Drug Administration (FDA) for clinical use of biostimulation lasers has

thus far not been granted for any therapeutical indication [23].

One of the major difficulties in low energy laser research is the lack of comparability between the various studies, since there is an enormous diversity in the physical laser parameters and dose regimens used. For the same reason, it seems virtually impossible to correlate effects on cellular function with effects in animal or human studies. Our starting point for this study was to test the effects of a commercially available laser device, which is being used in routine physiotherapeutical and dental practice throughout Europe, in an experimental in vitro setup at energy densities similar to those administered clinically. In clinical practice, the energy densities that are routinely applied to reduce inflammatory activity and to stimulate wound healing, generally do not exceed 10.0 J/cm^2 per irradiation [1].

As described above, cytokines play a pivotal role in such inflammatory and regenerative processes, and it thus seemed worthwhile to study the effects of low energy laser light on the release of these mediators by immunocompetent cells in vitro. Indeed, this notion was supported by several studies, reporting modulatory effects of low energy laser light on the release of specific cytokines by mononuclear cells in vitro. Funk and coworkers [24] investigated the effects of He-Ne laser irradiation on cytokine release by human peripheral blood mononuclear cells (PBMC) and found no induction of either IL-1α, TNFα, IL-2, or IFNγ by unstimulated cell cultures. However, when PBMC were stimulated with mitogens, such as LPS, following laser treatment, an increase of the secretion of these cytokines was observed at an energy density of 18.9 J/cm^2, whereas irradiation at even higher energy densities had inhibitory effects. In contrast, in the present study we were unable to demonstrate a modulatory effect on the release of TNFα, IL-6, and IL-8 by LPS-stimulated monocytes at much lower energy densities, similar to those applied clinically. Besides the discrepancy in energy densities used, a further difference between both studies is that, in our study, pure monocyte populations were used, whereas PBMC consist of monocytes as well as lymphocytes. A differential susceptibility to laser light between both cell types as well as interactions within such a mixed cell population during or after laser treatment might possibly explain the conflicting results between these studies.

Interestingly, Rossano et al. [25] have compared the effects of soft laser irradiation on purified monocytes and lymphocytes in culture. In their study, exposure to a single as well as a double irradiation strongly stimulated the release of IL-1α, TNFα, and IL-6 by monocytes, to the extent comparable with mitogen-induced cytokine release. In contrast, no interference with production of IL-4 and IFNγ by lymphocytes was observed, indeed indicating a difference in susceptibility to laser light between the two cell types. Nevertheless, the described stimulatory effects on monocyte-derived cytokines could not be confirmed by our data, but laser parameters may have differed considerably between our study and that of Rossano and coworkers [25]. Young et al. [26] suggested the release of hitherto unidentified soluble regulatory factors by the macrophage-like cell line U937 after irradiation at an energy density of 2.4 J/cm^2. Since this cell line is known to produce TNF-α and IL-6 [27], we subjected these cells to a similar laser treatment as described above for human monocytes. We found that a single laser treatment of 0.3, 3.0, or 9.0 J/cm^2 did not affect release of TNF-α, IL-6, and IL-8 by either unstimulated or stimulated U937 cells (data not shown). In this context, it is of interest that O'Kane et al. [28] recently reported the low energy laser-induced release of IL-6 by U937 cells at energy densities of 5.8 J/cm^2 and higher. At lower energy densities, ranging from 1.0–2.9 J/cm^2, no induction of IL-6 was detectable. In conclusion, uncertainty remains with regard to the effects of low energy laser on cytokine release by mononuclear cells, the relative importance of irradiation parameters such as wavelength, energy density, and power density being unclear at this time. Whether the potential effects of low energy laser on cytokine release are mediated through reciprocal interaction between different immunocompetent cell types remains another interesting issue to be addressed.

Using a similar in vitro experimental setup as described above for MΦ, we extended our study to vascular endothelial cells. By acting both as target and as source of cytokines, the vascular endothelium is of pivotal importance in leukocyte recruitment into inflammatory sites. In our hands, either single or repeated low energy laser irradiation did not induce release of IL-6 and IL-8 or de novo expression of E-selectin, ICAM-1, and VCAM-1. In addition, it failed to modify the response of HUVEC to stimulation with IL-1β or TNFα, as determined by these specific parameters. To our knowledge, this is the first study to investigate the effects of low energy laser irradi-

ation on the release of cytokines and the expression of specific adhesion molecules by human endothelial cells. Although it is not possible to relate our specific results obtained with endothelial cells to work by others, it is of significance that Colver and Priestly [29] were unable to detect any significant positive effect on endothelial cell proliferation in a study, similarly designed to assess the in vitro effects of a therapeutic clinical laser.

In summary, using a routine therapeutic laser at clinically relevant energy densities, we were unable to demonstrate a modulatory effect of low energy laser irradiation on the inflammatory function of two different immunocompetent cell types of human origin, i.e., monocytes and endothelial cells. The interpretation of these results in relation to observations reported by others is difficult due to differences in physical laser parameters. In our view, the relevance of experimental application of much higher energy densities than those used in this study is questionable. In this context, it must be taken into consideration that in the clinical setting, the minimal response dose at a certain penetration depth always will be lower than the energy density applied at the skin level. We conclude, therefore, that the results of this study do not provide a possible explanation for the biostimulative and anti-inflammatory effects attributed to low energy laser therapy.

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